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# Identification of Estrogenic Compounds in Fish Bile Using Bioassay-Directed Fractionation

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Conjugates of estrogenic chemicals, endogenous as well as xenobiotic, are mainly excreted via bile into the intestine. Therefore, measurement of estrogenic activity in bile yields useful information about an organism's internal exposure to (xeno-)estrogens. Although previous studies in The Netherlands have reported estrogenic activity in male fish bile, the contribution of natural hormones and xenobiotic substances to this activity is unknown. To identify compounds responsible for estrogenic activity in fish bile, we developed a bioassay-directed fractionation method for estrogenic chemicals. In this approach, the *in vitro* reporter gene assay ER-CALUX (Estrogen Responsive Chemical Activated Luciferase Gene Expression) was used to assess estrogenic activity in deconjugated bile samples and to direct RP-HPLC fractionation and chemical analysis (by GC-MS) of estrogenic compounds. The method was applied to bile from male breams (*Abramis brama*) collected at three locations in The Netherlands. At one of these locations, the River Dommel, extremely high levels of plasma vitellogenin and a high incidence of intersex gonads in these male breams have previously been observed, indicating the exposure to estrogens. In this study, the natural hormones 17 $\beta$ -estradiol, estrone, and estriol accounted for the majority of estrogenic activity in male bream bile. At the River Dommel, the synthetic contraceptive pill component ethynylestradiol was found in effective concentrations as well. The detected natural and synthetic hormones may be responsible for the estrogenic effects observed in wild bream from this location. Furthermore, a large number of xenobiotic chemicals was detected at relatively high levels in bile, including triclosan, chloroxylenol, and chlorophene. Although chloroxylenol was shown for the first time to be weakly estrogenic, these compounds did not contribute significantly to the estrogenic activity observed.

## Introduction

The presence of estrogenic compounds in the environment has attracted considerable attention because of the interference these xenobiotic as well as natural chemicals may exert on the normal functioning of human and wildlife

endocrine systems. Estrogenic compounds detected in the environment include, for example, the natural steroid hormones estrone (E1), 17 $\alpha$ -estradiol ( $\alpha$ -E2) and 17 $\beta$ -estradiol ( $\beta$ -E2), and estriol (E3) and the synthetic steroid and contraceptive 17 $\alpha$ -ethynylestradiol (EE2). Industrial chemicals with estrogenic properties have been detected as well, for example, bisphenol A (BPA; used to produce epoxy resins and polycarbonate plastics), the alkyl phenols 4-octylphenol (4-OP) and 4-nonylphenol (NP) and their mono- and diethoxylates (used mainly as polyethoxylates in industrial detergents and emulsifiers). Although endogenous hormones have been present in the environment for a very long time, the growing population and more intensive farming have raised the influx in aquatic systems (1). For The Netherlands, a country densely populated by both humans (16 million people on 45 000 km<sup>2</sup>) and livestock (18 million cattle, horses, pigs, and sheep), an emission of the natural steroids E1,  $\alpha$ - and  $\beta$ -E2 by humans and cattle of 10 kg/d, and about 50 g/d EE2 has been estimated (2). The annual estrogen excretion of farm animals in urine and faeces, which, unlike human waste, mostly does not pass sewage treatment plants, was estimated to reach 33 t in the European Union and about 49 t in the United States (reviewed by ref 1).

The presence of estrogenic compounds in the aquatic environment has been demonstrated numerous times (3–8). Exposure of fish to estrogens can lead to the induction of the precursor yolk protein vitellogenin in plasma of male fish (8, 9). Vitellogenin (VTG) levels in male fish blood plasma, which are usually negligible, can rise to concentrations in the tens of milligrams per milliliter range as a result of exposure to estrogens (10). VTG is therefore widely used as a biomarker of estrogenic exposure (11). Moreover, mainly in the United Kingdom, a high occurrence of intersexuality (a condition in which oocytes are formed in the testicular tissue) in wild populations of riverine fish has been observed (12). This was found to be consistent with exposure to hormonally active substances and associated with discharges from sewage treatment plants (STPs) that are known to contain estrogenic chemicals (13). In The Netherlands, an investigation of the occurrence and effects of estrogenic compounds in the aquatic environment (LOES) was recently performed (14). The investigation showed that almost all selected endocrine disrupting compounds (natural hormones, BPA, alkyl phenols and alkyl phenol ethoxylates, phthalates, and brominated flame retardants) were present at low concentrations (14). At some specific locations (especially in the vicinity of STPs), compounds were found at higher levels. Incidence of estrogenic effects in male fish was highest in regional surface waters that are strongly influenced by potential sources of emission of endocrine disrupting compounds. For example, in the River Dommel, a small river receiving relatively high amounts of STP effluent, extremely high plasma VTG concentrations and a high prevalence of intersexuality in male bream (*Abramis brama*), an abundant freshwater fish species in The Netherlands, were observed (14).

Previous research has shown that measurement of estrogenic compounds in fish bile may provide a useful indication of internal exposure to estrogens. Caged juvenile rainbow trout in STP effluent have been found to accumulate estrogenic compounds in their bile, which corresponded with large amounts of VTG in their plasma (15). Furthermore, a good correlation was found between total bile estrogenic activity and plasma vitellogenin induction in male bream (16). Bile is a suitable matrix to measure internal dose due to its role in biotransformation and elimination of com-

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pounds. In fish, estrogens are eliminated mainly by metabolic conversion to water-soluble metabolites. Oxidative metabolism of estrogens is catalyzed by cytochrome P450 enzymes in the liver (17). For conjugation of hydroxylated metabolites of steroids as well as xenobiotics, glucuronidation is likely to be the dominant pathway for biliary excretion. In rainbow trout, approximately 90% of estradiol is excreted as E2-glucuronide (17). For the xenoestrogen 4-*tert*-octylphenol it was observed that, after a 10-d exposure, 38% of the total accumulation in the body of rainbow trout was found back in the bile, present as glucuronides (18). A major pathway of elimination is excretion via bile into the intestines. In the intestines, estrogen conjugates may be broken down by intestinal bacteria, thereby eliciting the active parent compound. In the environment, estrogens are mostly found in their biologically active parent forms, probably due to bacterial hydrolysis of the excreted conjugates (3).

The present study was undertaken to elucidate the identity of compounds that contribute to the high internal levels of estrogenic activity in bream. Chemical analysis of the bile samples was not performed in the LOES study, and compounds responsible for the measured estrogenic activity in bile remained unknown. Therefore, in this study, a toxicity identification and evaluation (TIE) method that uses bioassay-directed fractionation was developed for estrogenic chemicals in fish bile. The estrogen responsive *in vitro* reporter gene assay ER-CALUX (19) was used to direct the fractionation and chemical analysis of extracts of deconjugated male fish bile. This method was applied to bile samples of male bream from three study locations of the Dutch LOES study. The natural hormone 17 $\beta$ -estradiol was identified as the main contributor to *in vitro* estrogenic activity in male fish bile.

## Materials and Methods

**Standards and Solvents.** High purity standards of  $\beta$ -E2, 17 $\beta$ -estradiol-3 $\beta$ -D-glucuronide ( $\beta$ -E2-gluc), E1,  $\alpha$ -E2, E3, EE2, 4-*tert*-OP, BPA, cholesterol, chloroxylenol, and chlorophene (all >98% pure, except chlorophene, which was 95% pure) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). NP was obtained from Acros Organics (Geel, Belgium), and triclosan was from Ciba Specialty Chemicals (Grenzach, Germany).

Water (H<sub>2</sub>O, HPLC analyzed), methanol (MeOH, HPLC gradient grade), and *n*-hexane (Ultra resi analyzed) were purchased from Mallinckrodt Baker (Deventer, The Netherlands). Dichloromethane (DCM, Suprasolv GC quality) was obtained from Merck (Darmstadt, Germany), ethyl acetate (EtAc) was bought from Fluka Chemie (Buchs, Switzerland), and dimethyl sulfoxide (DMSO, spectrofotometric grade 99.9%) was from Acros (Geel, Belgium).

**Efficiency of Deglucuronidation and Extraction of Estrogens in Bile.** Portions of 1 mL of water as well as pooled bile (obtained from nine male breams) were spiked with  $\beta$ -E2 (42.8 ng/mL, which represented ~6-fold background level, as established in unspiked bile) or  $\beta$ -E2-gluc (784.5 ng/mL ~100-fold background level) and subsequently in triplicate deconjugated in portions of 100  $\mu$ L with  $\beta$ -glucuronidase-sulfatase (from *H. pomatia*, 100  $\mu$ L of 400 U/mL dissolved in 0.2% NaCl; Sigma-Aldrich, Zwijndrecht, The Netherlands) as described before (16). To investigate the necessity of enzymatic deglucuronidation, similar experiments were performed in which the enzyme solution was replaced with 100  $\mu$ L of 0.2% NaCl. After deconjugation, one drop of 1 M HCl was added. Deconjugation products were extracted by liquid-liquid extraction with DCM or EtAc in order to determine the solvent yielding the best extraction efficiency. After addition of 2 mL of DCM (or EtAc), extraction tubes were vortexed for 1 min and centrifuged for 5 min at 1500 rpm. The DCM (or EtAc) phase was subsequently removed

and transferred to another test tube. The extraction was repeated to a total of three times, after which the collected DCM (or EtAc) phase was carefully evaporated to a small drop. This was quantitatively transferred into a conical vial and taken up in 70  $\mu$ L of DMSO. Glassware was prerinsed with DCM (or EtAc) for all extraction procedures. Efficiencies of deglucuronidation and extraction were determined by assessment of estrogenic activity in the samples with the ER-CALUX assay.

**ER-CALUX.** ER-CALUX-assay was performed with stably transfected T47D human breast cancer cells (T47D.Luc-cells) according to Legler et al. (19) with adaptations as described elsewhere (20). T47D.Luc-cells were obtained from Bio-Detection Systems B.V. (Amsterdam, The Netherlands). A concentration series of  $\beta$ -E2 was included on each plate. A sigmoidal standard curve [ $y = a_0 + a_1 / (1 + \exp(-(x - a_2) / a_3))$ ]; with  $y$  representing luciferase activity in relative light units and  $x$  representing the concentration of  $\beta$ -E2) was fitted using the software program Slidewrite 4.1. Bile samples were tested in triplicate. The luciferase activities of (dilutions of) bile samples were interpolated in the linear range of the  $\beta$ -E2 standard curve. To assess the estrogenic potency of  $\beta$ -E2-gluc, E1,  $\alpha$ -E2, E3, EE2, NP, 4-*tert*-OP, BPA, cholesterol, chloroxylenol, chlorophene, and triclosan, concentration series were prepared and tested in triplicate in at least two independent experiments. For  $\beta$ -E2 and the other active estrogens, half-maximum effective concentrations (EC<sub>50</sub>) were derived from the curves. For comparison of estrogenic potency between  $\beta$ -E2 and the other estrogens, the estrogenic activity of the estrogen X was expressed relative to that of  $\beta$ -E2 by calculation of the estradiol equivalence factor (EEF value) for X with the formula  $EEF_X = EC_{50(\beta-E2)} / EC_{50(X)}$ .

**Study Sites.** *Lake Bergumermeer.* Lake Bergumermeer is a lake of some 6 km<sup>2</sup> in the northern part of The Netherlands. The lake and its surroundings are destined to become a nature preservation area. On the southern side of the lake, there is some industrial activity. A power plant is situated on the northern side. Effluent of the STP of the small town of Bergum is discharged in the lake. This location was chosen as reference location. At this location female bream bile was also sampled, extracted, and tested for estrogenicity in the ER-CALUX assay.

*River Dommel.* The River Dommel is a small river originating in Belgium. It flows through an agricultural area, several small towns, and the city of Eindhoven into the River Dieze that runs into the Meuse. At Eindhoven, surface water from the Dommel and STP effluent mix at an approximate 1:1 ratio.

*Amsterdam North Sea Canal.* The North Sea Canal forms the connection between Amsterdam and its port in the west and the interior freshwater Lake Ysselmeer in the east. The canal, which flows through a heavily industrialized area, has a port and transport function and is used for discharging surplus water from several adjoining water board districts and STP effluent.

**Bile Sampling.** Methods of bream bile sampling have been described elsewhere (14). In brief, fish were captured in the fall of 1999, anaesthetised with MS 222 (3-aminobenzoic acid ethyl ester, Sigma Aldrich, Zwijndrecht, The Netherlands), and sacrificed. After the guts were removed, samples of bile fluid (typical volume about 2 mL) were taken with syringes. Samples were stored in ethanol-rinsed Eppendorf cups at -80 °C.

**Deconjugation and Extraction of Bile Samples.** Fish bile samples were pooled per sex per location. Portions of 1 mL of pooled bile were subsequently deconjugated with  $\beta$ -glucuronidase-sulfatase. Deconjugation products were extracted by liquid-liquid extraction with DCM as described above. The extracts were divided into two portions. Twenty percent of the extract was evaporated and dissolved in 70  $\mu$ L of DMSO for ER-CALUX measurements. The other 80% was evaporated



and taken up in 100  $\mu$ L MeOH:H<sub>2</sub>O (1:1 v/v) for fractionation by RP-HPLC.

**RP-HPLC Fractionation.** A mixture of 19 environmentally relevant compounds (toluene (Fluka Chemie, Buchs, Switzerland), 6 polycyclic aromatic hydrocarbons (PAHs: phenanthrene, fluoranthene, naphthalene, acenaphthylene, acenaphthene, fluorene; Promochem, Wesel, Germany), 3 polychlorinated biphenyls (PCBs: 2,2',4,5,5'-pentachlorobiphenyl (PCB 101), 2,2',3,4,4',5'-hexachlorobiphenyl (PCB 138), 2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180); Promochem, Wesel, Germany), 4 chlorobenzenes (1,2-dichlorobenzene, 1,3-dichlorobenzene, 1,2,4-trichlorobenzene, and 1,2,3,4-tetrachlorobenzene; Sigma-Aldrich, Steinheim, Germany), chloridazone, metalaxyl (Riedel de Haën, Seelze, Germany), benzothiazole (Sigma-Aldrich, Steinheim, Germany), octachlorostyrene, and *o,p'*-dichlorodiphenyldichloroethane (both from Dr. Ehrenstorfer, Augsburg, Germany) covering a wide range of log  $K_{ow}$  was prepared in MeOH. To 50  $\mu$ L of this mixture, 50  $\mu$ L of H<sub>2</sub>O was added, and the mixture was injected on a reversed phase high-pressure liquid chromatography system with a UV detector (Shimadzu, Duisburg, Germany,  $\lambda$  = 254 nm) at 22 °C. The system was equipped with a C<sub>18</sub> column (Vydac TP254, 5  $\mu$ m, 4.6  $\times$  250 mm). The mobile phase consisted initially of 50% MeOH and 50% H<sub>2</sub>O (1 mL/min) and changed linearly to 100% MeOH in 50 min according to ref 21. Retention times ( $T_{ret}$ ) were corrected for dead volume time ( $T_0$ ). Corrected values ( $T'_{ret}$ ) were correlated to log  $K_{ow}$  values to obtain a standard curve from which fraction intervals with a range of approximately one log  $K_{ow}$  unit were derived. Fraction border times were determined by adding  $T_0$  to the fraction interval times and correcting them for the volume of the tubing between detector and fraction collector. Subsequently, a mixture of eight known estrogenic compounds (BPA,  $\alpha$ -E2,  $\beta$ -E2, E1, E3, EE2, 4-*tert*-OP, and NP) in concentrations easily detectable with UV detection ( $\lambda$  = 254 nm) was injected. Retention times of the estrogens were derived from the obtained chromatogram. MeOH:H<sub>2</sub>O bile extracts were injected, and 10 fractions with a range of approximately 1 log  $K_{ow}$  unit each were collected. These fractions were dried with N<sub>2</sub> at 40 °C and split into three portions. Portion A (20% of the extract weight) was evaporated and dissolved in 70  $\mu$ L of DMSO for ER-CALUX measurements, Portion B (40%) was used for the analysis of known estrogens. Portion C (40%) was destined for general chemical screening using gas chromatography–mass spectrometry (GC–MS). After injection, the vial that had contained the MeOH:H<sub>2</sub>O extract was rinsed with *n*-hexane to dissolve the observed film of compounds presumably too nonpolar to dissolve in MeOH:H<sub>2</sub>O prior to fractionation. This fraction (the nonpolar residual fraction) was dissolved in 70  $\mu$ L of DMSO and tested for estrogenicity with ER-CALUX. Afterward, to enable chemical analysis of this nonpolar extract, this DMSO fraction was dissolved in water and nonpolar compounds were extracted with *n*-hexane. The nonpolar extract was evaporated to 100  $\mu$ L. To assess the repeatability of the partitioning of estrogenic compounds over the MeOH:H<sub>2</sub>O and the vial surface, pooled bile from location North Sea Canal was deglucuronidated, extracted, and dissolved in MeOH:H<sub>2</sub>O. The extract was removed, and the vial was rinsed with *n*-hexane to generate a new nonpolar residual extract. Both the nonpolar residual extract and the MeOH:H<sub>2</sub>O fraction were dissolved in DMSO and tested in ER-CALUX.

**Chemical Analysis.** *Hormones and Bisphenol A.* Portions B of the RP-HPLC fractions were evaporated to dryness at 55 °C and silylated with SIL A reagent (a mixture of (CH<sub>3</sub>)<sub>3</sub>-SiCl, (CH<sub>3</sub>)<sub>3</sub>SiNH<sub>2</sub>Si(CH<sub>3</sub>)<sub>3</sub>, and pyridine in a ratio of 1:3:9; Sigma-Aldrich, Zwijndrecht, The Netherlands) during 1 h at 55 °C. After evaporation, the residue was dissolved in *n*-hexane together with the internal standard PCB 103. The mixture was washed with water to remove byproducts that

could damage or contaminate the GC column or detector. The *n*-hexane phase was dried over a sodium sulfate column and collected in a GC conical vial. The *n*-hexane phase was evaporated to 100  $\mu$ L of which 3  $\mu$ L was splitlessly injected on the GC column.

Analysis was carried out on a GC–MS–MS (Varian CP3800 GC with a Varian Saturn 2200 ion trap detector), equipped with a 30 m Varian CP-SIL 8 CB low-bleed MS column (0.25 mm i.d., 0.25  $\mu$ m film thickness). The GC column was protected with a 2-m retention gap of deactivated fused silica 0.53 mm i.d. The temperature program was as follows: initial temperature 60 °C for 1 min, increasing linearly with 30 °C/min to 220 °C, then at a rate of 4 °C/min to 280 °C, and 50 °C/min to the final temperature at 300 °C, with a total runtime of 30 min. Ions for data acquisition in MS/MS mode were for  $\alpha$ -E2 and  $\beta$ -E2 derivatives  $m/z$  416/285+326, for EE2 derivative  $m/z$  425/193+231+303, for E1 derivative  $m/z$  342/257, and for E3 derivative  $m/z$  296+311+414+504. For BPA, only the ion with  $m/z$  = 357 was monitored. Quantification of hormones and BPA was performed by external calibration using a calibration series of standards of 10 different concentrations between 0 and 200 ng of compound/100  $\mu$ L of solvent. Standards were derivatized as described for the bile fractions.

**Chemical Screening.** Portions C of RP-HPLC fractions were evaporated until dryness and taken up in 100  $\mu$ L of *n*-hexane. Fractions and nonpolar residual extracts (3  $\mu$ L) were splitlessly injected on a GC–MSD (Agilent 6890 with a Agilent 5973 network quadrupole mass selective detector), equipped with a SGE-BPX5 column (25 m, 0.22 mm i.d., 0.3  $\mu$ m film thickness) and with helium (1 mL/min) as carrier gas, operated in full-scan mode ( $m/z$  50–650). Mass spectra were deconvoluted using the automated mass spectral deconvolution and identification system AMDIS and compared with reference spectra in the National Institute of Standards and Technology NIST main mass spectral database (NIST/EPA/NIH Mass Spectral Database, NIST 1992, 1998; Gaithersburg, MD) (match factor  $\geq$  80) for tentative identification. To confirm the identification of compounds, pure standards dissolved in *n*-hexane were injected for the comparison of retention time and Kovat's Retention Indices (KRI values) with those of the tentatively identified compounds in the extracts. Concentrations of individual compounds were semiquantified using the response of the pure standards.

## Results and Discussion

**Development of TIE Procedure.** The TIE procedure developed for the analysis of estrogenic compounds in fish bile (including deglucuronidation and extraction methods, RP-HPLC fractionation, chemical analysis, and screening) is shown schematically in Figure 1. The development of each step of the procedure and application to field samples are described below.

**Deglucuronidation and Extraction of Estrogen Conjugates in Bile.** Extraction of deconjugated  $\beta$ -E2 from water and bile was performed by liquid–liquid extraction with DCM or EtAc. Recoveries of estrogenic activity in the ER-CALUX assay are shown in Table 1. Both solvents gave high, comparable, and reproducible recoveries. DCM has a higher volatility than EtAc and is more easily evaporated. For these reasons, DCM was selected for the extraction of bile samples from the study sites.

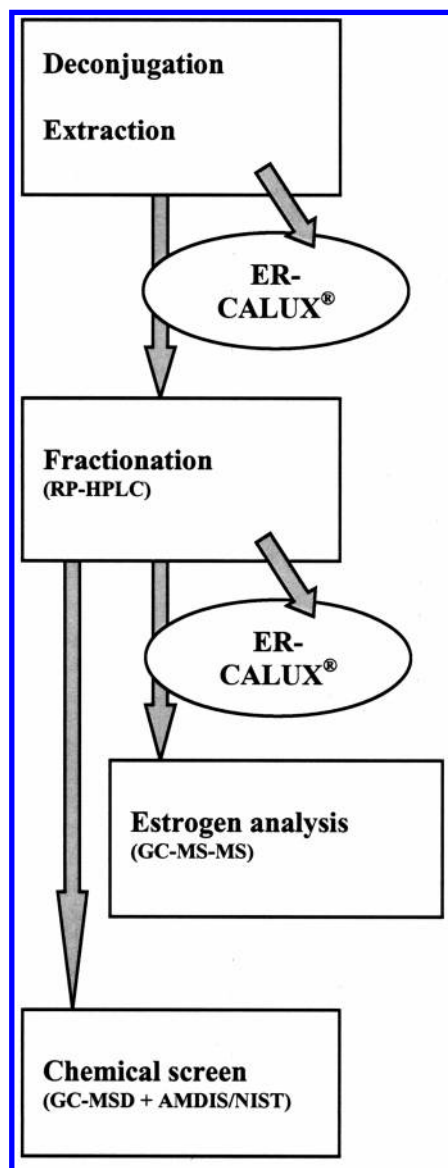
A matrix influence on the extraction was observed for bile, as deviations from 100% recovery were larger in bile than in water (Table 1). The extraction efficiency of deconjugated  $\beta$ -E2-gluc was higher than that of  $\beta$ -E2. Although the reason for this phenomenon is unclear, it may be due to the much higher spiking level of  $\beta$ -E2-gluc in the samples.

Deglucuronidation efficiency was tested by comparing recoveries of deconjugated  $\beta$ -E2-gluc that had been added

**TABLE 1. Recovery (Average  $\pm$  SD) of Estrogenic Activity from Dichloromethane or Ethyl Acetate Extracts of 17 $\beta$ -Estradiol or 17 $\beta$ -Estradiol-3- $\beta$ -D-glucuronide Spiked Male Bream Bile and Water without or after Deglucuronidation, Measured with ER-CALUX<sup>a</sup>**

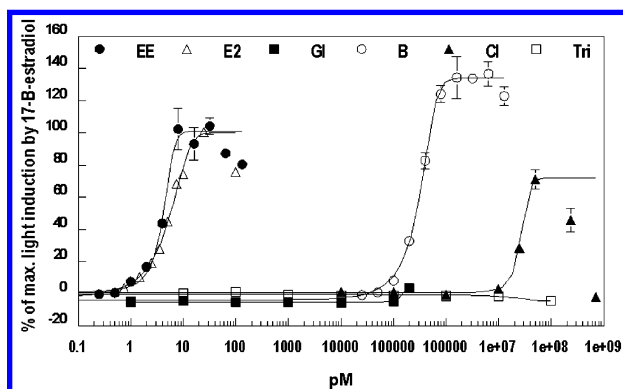
	DCM				EtAc			
	with enzyme (ng of EEQ/mL)	recovery (%)	without enzyme (ng of EEQ/mL)	recovery (%)	with enzyme (ng of EEQ/mL)	recovery (%)	without enzyme (ng of EEQ/mL)	recovery (%)
water	0.0 $\pm$ 0.0	—	0.0 $\pm$ 0.0	—	0.0 $\pm$ 0.0	—	0.0 $\pm$ 0.0	—
water + $\beta$ -E2	42.3 $\pm$ 0.2	98.8 $\pm$ 0.5	na	na	39.9 $\pm$ 4.0	93.2 $\pm$ 9.3	na	na
water + $\beta$ -E2-gluc	826 $\pm$ 137	105 $\pm$ 17	0.3 $\pm$ 0.1	0.0 $\pm$ 0.0	886 $\pm$ 234	113 $\pm$ 30	2.5 $\pm$ 1.8	0.3 $\pm$ 0.2
bile	7.9 $\pm$ 0.3	—	0.5 $\pm$ 0.1	—	10.4 $\pm$ 2.1	—	na	—
bile + $\beta$ -E2	36.3 $\pm$ 3.0	72.3 $\pm$ 6.0	na	na	39.8 $\pm$ 5.7	74.8 $\pm$ 10.7	na	na
bile + $\beta$ -E2-gluc	736 $\pm$ 55	92.9 $\pm$ 6.9	114 $\pm$ 26	14.4 $\pm$ 3.3	1112 $\pm$ 145	140 $\pm$ 24	na	na

<sup>a</sup> All experiments were performed in triplicate. Recoveries in bile were corrected for background levels. Abbreviations: DCM, dichloromethane; EtAc, ethyl acetate; EEQ, estradiol equivalents;  $\beta$ -E2, 17 $\beta$ -estradiol;  $\beta$ -E2-gluc, 17 $\beta$ -estradiol-3- $\beta$ -D-glucuronide; —, recovery not applicable; na, not assessed.



**FIGURE 1. Sample treatment scheme for the toxicity identification and evaluation of estrogenic compounds in fish bile.**

as  $\beta$ -E2-gluc prior to deglucuronidation to water or bile in the presence or absence of  $\beta$ -glucuronidase-sulfatase (Table 1). In water, as in bile, all  $\beta$ -E2-gluc was deconjugated, which confirmed the suitability of this method for further use. In bile, some estrogenic activity was observed after deglucuronidation of  $\beta$ -E2-gluc in the absence of added enzyme. Because  $\beta$ -E2-gluc does not show any estrogenic activity



**FIGURE 2. Estrogenic potency of ethynylestradiol (EE), 17 $\beta$ -estradiol (E2), 17 $\beta$ -estradiol-3- $\beta$ -D-glucuronide (GI), bisphenol A (B), chloroxylenol (Cl) and triclosan (Tri) in the in vitro ER-CALUX assay.**

(Figure 2), this suggests the occurrence of some endogenous hydrolysis activity in bile. This was not observed for water. Estrogenic activity in unspiked deconjugated male bream bile was around 17-fold higher than in untreated bile. In real life, bream are exposed to estrogens, for example, via consumption of food and via water passing the gills. Estrogens can be conjugated in the liver and excreted via the bile shortly after intake or can go into circulation before being transported to the liver to be conjugated and excreted via the bile. Therefore, after deglucuronidation, the estrogenic activity in bile reflects the maximum bioavailable fraction.

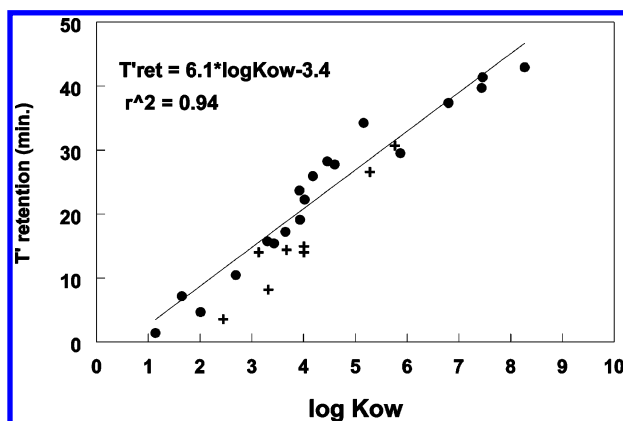
**RP-HPLC Fractionation.** The chromatographic behavior of a mixture of compounds of different polarity under the RP-HPLC conditions used was estimated with a mixture of PAHs, PCBs, chlorobenzenes, and several pesticides. As expected according to ref 21, strong correlations were found between the capacity factor  $((T_{\text{ret}} - T_0)/T_0)$  and  $\log K_{\text{ow}}$  ( $r^2 = 0.93$ ) and between  $T'_{\text{ret}}$  and  $\log K_{\text{ow}}$  ( $r^2 = 0.94$ ; Figure 3). This enabled the collection of fractions of bile extract according to decreasing polarity. Selected estrogenic compounds (represented by + in Figure 3) showed, although some of them eluted relatively early, chromatographic behavior comparable with that of the components in the standard mixture and could thus be fractionated well by this method. Some compounds (e.g., BPA and  $\beta$ -E2, having retention times very close to the fraction borders) eluted in neighboring fractions. Estimated  $T_{\text{ret}}$ , fractions of elution, and examples of  $\log K_{\text{ow}}$  values from literature (22–25) of the estrogenic compounds are given in Table 2.

To enable comparison of estrogenic activity measured in the ER-CALUX assay and concentrations of selected estrogens detected with GC-MS-MS, pure standards were tested in the ER-CALUX assay. Examples of the obtained dose-response curves are shown in Figure 2.  $\beta$ -E2 and EE2 were the most potent estrogens tested in this study. Interestingly,

**TABLE 2. Retention Time ( $T_{\text{ret}}$ ), Fraction of Elution during HPLC Fractionation, Log  $K_{\text{ow}}$ , and Estrogenic Potencies of Eight Selected Estrogenic Compounds<sup>a</sup>**

compound	$T_{\text{ret}}$ (min)	fraction (log $K_{\text{ow}}$ ) <sup>b</sup>	examples of log $K_{\text{ow}}$ values from literature	EEF (in vitro ER-CALUX)
E3	6.44	1–2 (1.14)	2.45 <sup>c</sup> , 2.81 <sup>d</sup>	0.13
BPA	11.09	1–2, 2–3 (1.90)	3.32 <sup>c</sup> , 3.64 <sup>d</sup>	1.13 E-5
E1	16.91	2–3 (2.86)	3.13 <sup>c</sup> , 3.43 <sup>d</sup>	0.12
$\alpha$ -E2	16.91	2–3 (2.86)	4.01 <sup>c</sup> , 3.94 <sup>d</sup>	0.01
EE2	17.31	2–3 (2.92)	3.67 <sup>c</sup> , 4.12 <sup>d</sup>	1.12
$\beta$ -E2	17.84	2–3, 3–4 (3.01)	4.01 <sup>c</sup> , 3.92 <sup>d</sup> , 3.10 <sup>e</sup>	1
4-tert-OP	29.45	4–5, 5–6 (4.91)	5.28 <sup>d</sup> , 4.12 <sup>f</sup>	7.3 E-5
NP	33.60	5–6 (5.59)	5.76 <sup>g</sup> , 4.48 <sup>f</sup> , 5.99 <sup>d</sup>	3.7 E-5

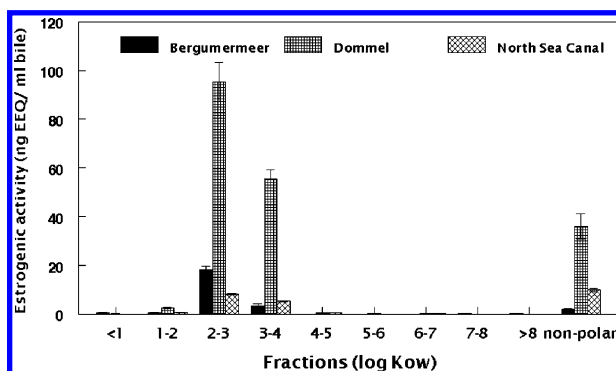
<sup>a</sup> E3, estriol; BPA, bisphenol A; E1, estrone;  $\alpha$ -E2, 17 $\alpha$ -estradiol; EE2, 17 $\alpha$ -ethynylestradiol;  $\beta$ -E2, 17 $\beta$ -estradiol; 4-tert-OP, 4-tert-octylphenol; NP, 4-nonyl phenol; EEF, estradiol equivalence factor (half-maximum effect concentration of  $\beta$ -E2 divided by half-maximum effect concentration of compound). <sup>b</sup> Values in parentheses indicate peak maxima (interpolated log  $K_{\text{ow}}$  values according to correlation shown in Figure 3). <sup>c</sup> Experimental value according to ref 22. <sup>d</sup> Estimated value according to EpiSuite Kowwin v1.67, U.S. EPA. <sup>e</sup> Experimental value according to ref 23. <sup>f</sup> Experimental value according to ref 24. <sup>g</sup> Experimental value according to ref 25.



**FIGURE 3. Relationship between corrected retention time ( $T'_{\text{ret}}$  = retention time – dead volume time) and log  $K_{\text{ow}}$  of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, chlorobenzenes, and several pesticides (all marked with dots) used to calibrate RP-HPLC elution conditions for fractionation of bile extracts ( $r^2$  = 0.94).  $T'_{\text{ret}}$  of selected estrogens with literature log  $K_{\text{ow}}$  values first mentioned in Table 2 are represented by +.**

BPA showed supramaximal response in the ER-CALUX assay as compared with  $\beta$ -E2. This might, for example, be due to a stimulation by BPA of the production of estrogen receptor. However, this does not affect the  $\text{EC}_{50}$  to a great extent. Relative estrogenic potencies derived from  $\text{EC}_{50}$  values of compounds used in the development of the fractionation method are given in Table 2.

**Application to Study Sites. Estrogenic Activity in Whole Extracts and Polar Fractions.** Although male fish are known to have estrogenic hormones, their basal concentrations are much lower than those in females. To confirm this, we assessed estrogenic activity in deconjugated male as well as female total bile extract from reference site Lake Bergumermeer. As expected, estrogenic activity in male bile ( $24.7 \pm 2.0$  ng of EEQ/mL of bile) was much lower than in female bile ( $353 \pm 17$  ng of EEQ/mL of bile). The TIE procedure was then applied to pooled male bile samples from three locations. Large differences in estrogenic activity as measured in the ER-CALUX assay in whole extracts of male bile were observed between the three study locations: Lake Bergumermeer  $24.7 \pm 2.0$ , River Dommel  $235 \pm 14$ , and North Sea Canal  $35.6 \pm 2.2$  ng of EEQ/mL of bile. Remarkably, fish bile from the River Dommel showed estrogenic activity in males almost at a female level of 1 order of magnitude higher than at the other locations. The River Dommel was the only location showing considerable vitellogenin induction (weighted average for this pool of samples 53.2 mg of VTG/mL, both other locations:  $\leq 0.5$  E-3 mg of VTG/mL (recalculation of data



**FIGURE 4. Distribution of estrogenic activity, expressed in estradiol equivalents (EEQ), in RP-HPLC fractions of male bream bile extracts.**

from ref 14). Also, intersex gonads (oocytes inside the seminiferous tubules) were observed in 33% of the male breams from this location (14). The correlation between high responses of bile samples in the estrogen receptor mediated ER-CALUX assay, VTG induction, and intersex suggests a common cause of events (i.e., exposure to estrogens; 13). Indeed, the role of the estrogen receptor in VTG induction has been established (26), and a number of studies have shown the correlation between VTG and effects on reproductive tissues in fish (27, 28).

**Fractionation of Estrogenic Activity.** To determine the nature of the estrogenic compounds in bile, whole extracts were fractionated as described above. The distribution of estrogenic activity over the fractions after RP-HPLC fractionation is shown in Figure 4. Most estrogenic activity was found in fractions of log  $K_{\text{ow}}$  2–3 and 3–4 (between  $8.3 \pm 0.3$  ng of EEQ/mL in for North Sea Canal and  $95.4 \pm 7.9$  ng of EEQ/mL for River Dommel, both in fraction log  $K_{\text{ow}}$  2–3, see Table 3). Unexpectedly, the nonpolar residual fraction, obtained by rinsing the vial that had contained the MeOH: H<sub>2</sub>O extract prior to injection on HPLC with *n*-hexane, contained considerable estrogenic activity as well. Estrogenic activities measured in these residual fractions were  $2.1 \pm 0.1$  ng of EEQ/mL of bile for Lake Bergumermeer,  $36.0 \pm 5.2$  ng of EEQ/mL of bile for River Dommel and  $9.9 \pm 0.7$  ng of EEQ/mL of bile for the North Sea Canal. The average recovery of estrogenic activity during fractionation, calculated by dividing the sum of the activity in all fractions by the activity in whole extract, was high ( $82 \pm 22\%$ ), indicating only small losses of estrogenic activity occur during fractionation.

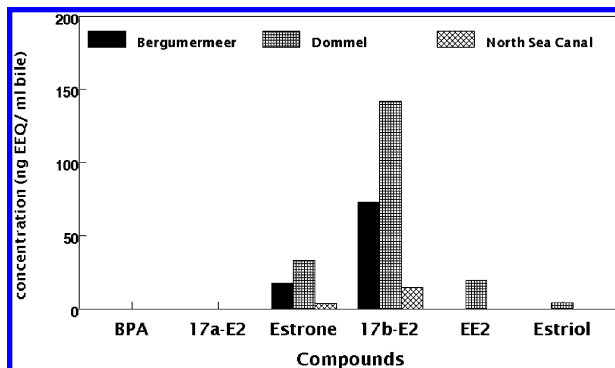
**Target Analysis of Known Estrogens in Polar Fractions.** Fractions with log  $K_{\text{ow}}$  <1, 1–2, 2–3, and 3–4 were analyzed with GC–MS–MS for the presence of the natural and synthetic hormones and BPA. Detected concentrations of compounds

**TABLE 3. Estrogenic Compounds Identified in Bile Extract Fractions and Associated Calculated and Measured Estrogenic Activities<sup>a</sup>**

	fraction log $K_{ow}$ <1 (ng of EEQ/mL)	log $K_{ow}$ 1–2 (ng of EEQ/mL)	log $K_{ow}$ 2–3 (ng of EEQ/mL)	log $K_{ow}$ 3–4 (ng of EEQ/mL)	sum fractions log $K_{ow}$ <1–3–4 (ng of EEQ/mL)
<b>Lake Bergumermeer</b>					
BPA	0.0 (3.9)	0.0 (28.5)	—	—	0.0 (32.4)
$\alpha$ -E2	—	—	0.1 (7.7)	—	0.1 (7.7)
E1	—	—	17.8 (149.3)	—	17.8 (149.3)
$\beta$ -E2	—	—	73.1 (73.1)	—	73.1 (73.1)
EE2	—	—	—	—	—
E3	—	—	—	—	—
sum GC <sup>b</sup>	0.0	0.0	91.0	0.0	91.0
ER-CALUX <sup>c</sup>	0.6	0.7	18.4	3.3	22.9
GC/ER-CALUX (%) <sup>d</sup>	0.0	0.0	494.6	0.0	395.6
GC/ER-CALUX <sub>total</sub> (%) <sup>e</sup>	0.0	0.0	356.9	0.0	356.9
<b>River Dommel</b>					
BPA	—	0.0 (1937.6)	0.0 (42.7)	—	0.0 (1980.3)
$\alpha$ -E2	—	—	0.1 (10.1)	—	0.1 (10.1)
E1	—	—	33.3 (279.8)	—	33.3 (279.8)
$\beta$ -E2	—	—	83.3 (83.3)	58.7 (58.7)	142.0 (142.0)
EE2	—	—	19.5 (17.4)	—	19.5 (17.4)
E3	—	0.7 (5.4)	4.1 (31.7)	—	4.8 (37.1)
sum GC <sup>b</sup>	0.0	0.7	140.3	58.7	199.7
ER-CALUX <sup>c</sup>	0.1	2.5	95.4	55.5	153.5
GC/ER-CALUX (%) <sup>d</sup>	0.0	28.0	147.1	105.8	130.1
GC/ER-CALUX <sub>total</sub> (%) <sup>e</sup>	0.0	0.4	73.6	30.8	104.8
<b>North Sea Canal</b>					
BPA	0.0 (4.3)	0.0 (3035.8)	0.0 (42.2)	—	0.0 (3082.3)
$\alpha$ -E2	—	—	—	—	—
E1	—	—	3.6 (30.1)	—	3.6 (30.1)
$\beta$ -E2	—	—	8.1 (8.1)	6.7 (6.7)	14.8 (14.8)
EE2	—	—	—	—	—
E3	—	—	—	—	—
sum GC <sup>b</sup>	0.0	0.0	11.7	6.7	18.4
ER-CALUX <sup>c</sup>	0.0	0.8	8.3	5.2	14.3
GC/ER-CALUX (%) <sup>d</sup>	1.8	4.5	141.0	128.8	128.7
GC/ER-CALUX <sub>total</sub> (%) <sup>e</sup>	0.0	0.1	47.2	27.0	74.2

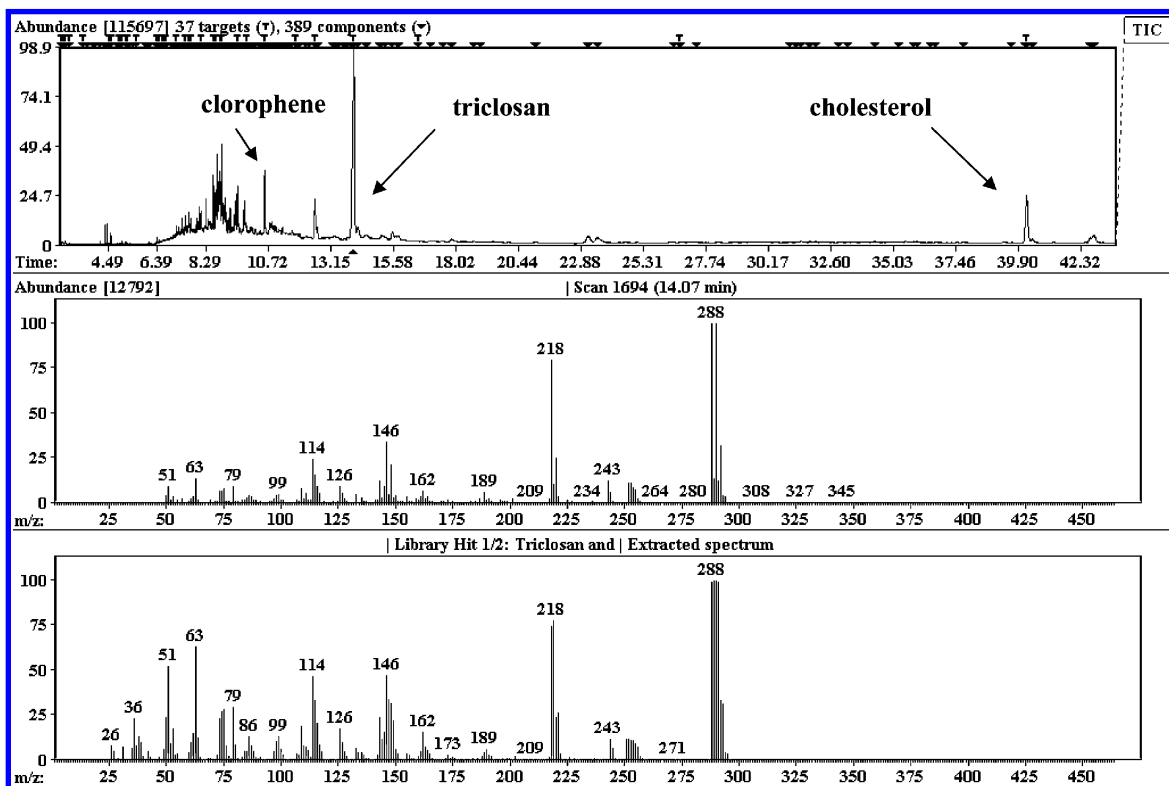
<sup>a</sup> All concentrations refer to the compounds measured in their derivatized forms and are expressed as estrogenic equivalents calculated by multiplying concentrations with the estrogenic equivalence factor to ng of EEQ/mL of bile. Concentrations of individual compounds (ng of compound/mL of bile) are given in parentheses. — indicates below limit of detection (LOD). LOD (ng/100  $\mu$ L GC-MS-MS extract) = 0.3 (BPA); 1.5 (17- $\alpha$ -E2); 1.5 (E1); 0.5 (17- $\beta$ -E2); 1.5 (EE2); 1.0 (E3). <sup>b</sup> Sum of calculated estrogenic activity in ng of EEQ/mL of bile of all chemically identified compounds per fraction. <sup>c</sup> Estrogenic activity measured in the ER-CALUX assay in ng of EEQ/mL of bile per fraction. <sup>d</sup> Estrogenic activity in ng of EEQ/mL of bile according to GC analysis per fraction divided by estrogenic activity in the same fraction in the ER-CALUX. <sup>e</sup> Estrogenic activity in ng of EEQ/mL of bile according to GC analysis per fraction divided by the sum of estrogenic activity in the ER-CALUX in all fractions, including the nonpolar residual fraction.

are presented in Table 3. As the estrogenic activity in fractions with log  $K_{ow}$  4–5 and 5–6 was negligible, it was concluded that 4-*tert*-OP and NP were not present in active amounts and therefore dedicated 4-*tert*-OP and NP analyses were not performed. To enable comparison with estrogenic activities in the ER-CALUX bioassay, all GC-measured concentrations were multiplied with their respective EEQ values (Table 2) to derive estrogenic activities caused by the presence of each individual compound. This approach is based on the concept of concentration addition as introduced in 1926 by Loewe and Muischnek and evaluated for estrogenic compounds by Kortenkamp and Altenburger (29). For each location, estrogenic activities are summed up for each fraction (columns in Table 3) and each compound (rows in Table 3). All analyzed compounds were detected in bile. In particular, BPA was found in considerable concentrations (up to 3  $\mu$ g/mL at location River Dommel). However, due to its low estrogenic potency (EEF = 1.13 E–5), its contribution to the measured estrogenic activity was negligible. For all three locations, most of the activity was accounted for by  $\beta$ -E2. This is shown in Figure 5. The activity of  $\beta$ -E2 was found in fractions log  $K_{ow}$  2–3 and 3–4. Its metabolite E1 was present in higher concentrations (about 280 ng/mL at location River Dommel) but, being less estrogenic than its parent compound (EEF = 0.12), contributed less to the total activity. The less stable metabolite E3 and the synthetic estrogen EE2 were found

**FIGURE 5. Concentrations of identified estrogenic compounds in fractions of male bream bile extracts, expressed as estradiol equivalents (EEQ), based on concentrations of derivatized compounds measured with GC-MS-MS.**

only in bile from River Dommel, albeit at relatively high concentrations (about 37 and 17 ng/mL, respectively) (Table 3). Our results for location River Dommel are in good agreement with the results obtained by Larsson et al. (15), who reported the presence of  $\beta$ -E2, E1, EE2, BPA, and NP in the bile of caged juvenile rainbow trout exposed to diluted sewage effluent water in Sweden, albeit at concentrations





**FIGURE 6.** GC-MSD chromatogram of the nonpolar residual fraction of male bream bile from the River Dommel (top), mass spectrum at 14.07 min (middle) with the by AMDIS deconvoluted spectrum, and National Institute of Standards and Technology reference spectrum of the adjudged substance triclosan (bottom).

on average about 1 order of magnitude higher than those at River Dommel.

In all three locations, measured compounds were present in concentrations high enough to explain the estrogenic activity in the four fractions analyzed with GC-MS-MS, with  $\beta$ -E2 as major contributor. For location Lake Bergumermeer, the total chemically identified estrogenic activity (91.0 ng of EEQ/mL) was almost 4-fold higher than the activity found in the ER-CALUX assay (18.4 ng of EEQ/mL). One of the explanations for this phenomenon could be the presence of antiestrogenic compounds in fraction log  $K_{ow}$  2–3 at this location. However, when tested together with  $\beta$ -E2 (results not shown), no anti-estrogenic activity in the ER-CALUX-assay was found in this fraction.

**Identification of Unknown Compounds in Nonpolar Residual Fractions.** The estrogenic activity in the four polar fractions was explained by the presence of the compounds measured with GC-MS-MS (Table 3). However, for locations River Dommel and North Sea Canal, considerable estrogenic activity in the ER-CALUX of an unknown chemical nature was found in the nonpolar residual fractions obtained by rinsing the vials that had contained the MeOH:H<sub>2</sub>O extracts used for fractionation with *n*-hexane. To identify chemical compounds present in these residual DMSO fractions, chemicals were re-extracted with *n*-hexane and a general chemical screen with GC-MSD was performed. Hundreds of different spectra were deconvoluted for the nonpolar extracts of the three locations. The presence of many (branched and linear) alkanes, oxidized alkanes, and alkanolic acids was suggested. Furthermore, several phenolic compounds were identified. In a retention time interval between 8 and 9.5 min, numerous alkylphenolic substances and their isomers were tentatively identified. In the nonpolar residual fraction from the River Dommel, for example, 4-*tert*-OP (~0.1  $\mu$ g/mL of bile) was found. Furthermore, the chlorinated phenolic disinfectants clorophene (o-benzyl-*p*-chlorophenol,

~7  $\mu$ g/mL, see Figure 6) and chloroxylenol (4-chloro-3,5-dimethylphenol, ~0.1  $\mu$ g/mL) were identified in this extract.

Phthalates (especially diethylhexylphthalate with  $m/z$  = 149 as major peak with  $T_{ret}$  = 29.19 min and KRI = 2534) were found showing considerable responses. However, as phthalates are notorious for contamination of samples during clean up in the laboratory, it is doubtful if this compound really originates from the bile, especially because it also was found in GC-MSD screens from many more polar fractions obtained by RP-HPLC. The fatty steroid cholesterol was detected in nonpolar residual fractions from all locations (see Figure 6 for the River Dommel). Concentrations were roughly estimated to be around 230  $\mu$ g/mL of bile for North Sea Canal and 20  $\mu$ g/mL of bile for River Dommel. In extract from the North Sea Canal, several other steroidal compounds besides cholesterol with  $T_{ret}$  between 36 and 43 min were tentatively identified as well (e.g., the plant sterol campesterol and the faecal sterol epicholesterol). Although the identification of these compounds could not be confirmed because standards were not available at our laboratories, the presence of these endogenous and other steroids in bile is not surprising as biliary secretion is a major excretion route for these substances.

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) was identified in the nonpolar residual fraction from locations River Dommel and North Sea Canal. The chromatogram peak, spectrum, deconvoluted spectrum, and library spectrum of triclosan are shown in Figure 6. Concentrations were about 14  $\mu$ g/mL of bile for North Sea Canal and 80  $\mu$ g/mL of bile for River Dommel. This diphenyl ether is commonly used as potent antibacterial and antifungal agent in consumer products as toothpastes, detergents, household sponges, socks, and underwear. The occurrence of triclosan in the environment has been reported before by Boyd et al. (30) for surface water and by Adolfsson-Erici et al. (31) for human breast milk, wastewater, sewage sludge, and fish bile. Our



results reconfirm that this compound eventually enters the aquatic environment, where it accumulates in fish bile.

To investigate if one or more of the identified compounds could be responsible for the estrogenic activity in the nonpolar residual fractions, concentration curves of identified compounds were tested for estrogenicity in the ER-CALUX assay. Chloroxylenol was found to be weakly estrogenic ( $EEF = 2.35 \times 10^{-7}$ ), as shown in the concentration response curve in Figure 2. Concentrations tested higher than  $50 \mu\text{M}$  caused visible cytotoxicity. To our knowledge, this is the first time the estrogenic activity of this compound has been shown. Although this antimicrobial agent has been widely used for years (e.g., as a disinfectant cleaning agent and preservative in pharmaceutical and cosmetic products; 32), no reports on the occurrence of chloroxylenol in the environment could be found. Cholesterol, triclosan, and chlorophene did not show any estrogenic behavior in concentrations up to  $0.1 \text{ mM}$  (for triclosan shown in Figure 2), indicating that these compounds are not or very weakly estrogenic ( $EEF < 5 \times 10^{-8}$ ) and not able to explain the activity measured. The concentrations in the nonpolar residual fraction of chloroxylenol and alkylphenols and phthalates, the only other identified compounds known to be (weakly) estrogenic, were too low to account for the estrogenic activity found. It is possible that trace amounts of active estrogens were lost during the transfer of the extract from DMSO to *n*-hexane for GC-MS-MS analysis. Indeed, the extraction of  $\beta$ -E2 dissolved in DMSO resulted in a mere 24% recovery (data not shown).

A closer look at the polarity of the compounds detected in the nonpolar fraction, revealed that, at the moment the total bile extract was taken up into MeOH:H<sub>2</sub>O prior to the HPLC-fractionation, a partitioning took place between the polar MeOH:H<sub>2</sub>O phase and the residual liquid layer remaining on the vial surface. Compounds with higher  $\log K_{ow}$  are mostly found in the nonpolar residual fraction. However, some compounds are found in both their appropriate RP-HPLC fraction (e.g., triclosan ( $\log K_{ow} = 4.76$ , fraction  $\log K_{ow} 4-5$ ), chlorophene ( $\log K_{ow} = 4.18$ , fraction  $\log K_{ow} 3-4$ ), and cholesterol ( $\log K_{ow} = 8.74$ , found in fraction  $\log K_{ow} > 8$ )) and in the nonpolar residual fraction. For location North Sea Canal, 40% of the total (HPLC fractions + nonpolar residual fraction) estrogenic activity was found in the nonpolar residual fraction. To investigate the repeatability of the partitioning over the MeOH:H<sub>2</sub>O and the vial surface, this step was repeated with freshly prepared bile extract from the North Sea Canal. Total estrogenic activity ( $29.4 \pm 0.7 \text{ ng of EEQ/mL of bile}$ ) was in good agreement with the total activity measured in the TIE-study ( $24.8 \pm 1.4 \text{ ng of EEQ/mL of bile}$ ). However, this time 94% of the total estrogenic activity was found in the MeOH:H<sub>2</sub>O fraction instead of 60% in the TIE study. As the analysis of total estrogenic activity proved to be repeatable, no activity was lost during extraction and fractionation experiments. Only the partitioning of active compounds between the responsive fractions differed. Clearly, the solvent transfer of chemicals of varying polarities as demonstrated by the transfer of bile extract from DCM to MeOH:H<sub>2</sub>O is a delicate process that should always be checked. However, the difference in partitioning does not influence the compounds detected, the extent to which they can explain estrogenic activity in their own fractions, the comparison of total estrogenic activity between locations, nor the interpretation concerning elevated VTG levels and intersex prevalence.

**Methodological Considerations.** The TIE method developed was successfully applied to identify compounds responsible for *in vitro* estrogenic activity in male bream bile. Because of bioconcentration of estrogenic compounds in this matrix, only  $10 \mu\text{L}$  of bile is needed to detect and quantify estrogenic activity in the ER-CALUX assay. Another advantage of the use of bile in this study is the relatively easy

extraction and low demand on clean up for the ER-CALUX assay and GC analysis. RP-HPLC fractionation led to a good separation of compounds according to their polarity. The necessity to dissolve the extract in a rather polar solvent mixture is inherent to the chosen RP-HPLC fractionation method. Our work shows that one should be aware of the presence of more nonpolar estrogenic compounds, which are incapable of completely dissolving in MeOH:H<sub>2</sub>O. Therefore, more attention should be given to methods for the investigation of the chemical nature of more nonpolar estrogenic compounds. Due to the reduction of complexity of the fractions as compared to the total extract, further cleanup to enable GC analysis was not necessary. The chemical screening performed with GC-MSD and AMDIS software has shown to be a powerful tool for the tentative identification of compounds. The concomitant use of a sensitive GC-MS-MS method for estrogenic hormones enabled us to investigate the contribution of the natural and synthetic estrogenic hormones at environmentally relevant levels. The combination of these two chemical analytical techniques allows for analysis of the very potent known natural and synthetic estrogens and the wide range of less potent industrial estrogens present in environmental samples.

**Causative Compounds.** This study showed that, in all three locations, the majority of the *in vitro* estrogenic activity could be explained by the presence of  $\beta$ -E2. The occurrence of unnaturally high concentrations of this hormone in male bile could at least have two causes. The first one is direct exposure of fish in the environment to  $\beta$ -E2, leading to uptake and bioconcentration of  $\beta$ -E2 in bile. Indeed, the occurrence of estrogenic hormones in surface waters and STP effluents in nanograms per liter concentrations has been widely reported (3, 5, 8, 14, 33). Another possibility is exposure to other endocrine disrupting compounds with the potential to disturb the metabolism of endogenous  $\beta$ -E2, for example, by inducing aromatase activity (34) or inhibiting elimination of  $\beta$ -E2 (35).

High biliary estrogens identified in this study correlated well with effects in the bream sampled, including elevated VTG plasma levels and intersexuality. However, one should be aware of the indirectness of the analysis of endocrine disruption causing compounds in this matrix because effects are not expected to take place in bile but in target organs as gonads and liver. In addition, caution should be taken when drawing conclusions about the relative contribution of  $\beta$ -E2 and other estrogens present to the effects observed in bream based on *in vitro* measurements. *In vitro* assays cannot completely reflect complex *in vivo* events, such as bioavailability and toxicokinetics of a compound. Therefore, considerable differences between *in vitro* and *in vivo* estrogenic potencies of compounds have been reported (36–38). For example, although the estrogenic potencies of pure standards of  $\beta$ -E2 and EE2 in the *in vitro* ER-CALUX assay are comparable (36) (Figure 2); EE2 has been shown to be a 4 times more potent VTG inducer than  $\beta$ -E2 in zebrafish (39) and even a hundred times more estrogenic than  $\beta$ -E2 in an *in vivo* transgenic zebrafish assay (36). This suggests that the involvement of EE2 in the generation of estrogenic effects is larger than expected based on *in vitro* measurements. Remarkably, the River Dommel, the only location with elevated VTG plasma levels and intersexuality (14), is also the only location with detectable EE2 concentrations in bile.

Estrogenic activity in bile reflects the animal's internal dose of estrogens. Therefore, measurement of estrogenic compounds in bile can be a suitable approach to reduce the gap between *in vitro* and *in vivo* measurements.

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